PATENT
GAUTVIK 3.0-001 FWC CIP CONT FWC DIV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of
Gautvik et al.

Serial No. 08/340,664

Filed: November 16, 1994

For: PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS

Group Art Unit 1812

Examiner: L. Spector

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF KAARE M. GAUTVIK, M.D.
PURSUANT TO 37 C.F.R. § 1.132

Sir:

I, KAARE M. GAUTVIK, declare as follows:

1. I am a coinventor of the above-captioned application.

2. I am a citizen of Norway residing at Bregnevn 3, 0875 Oslo, Norway. I am fluent in English. My curriculum vitae is attached hereto as exhibit A.

3. Throughout the 1980's, and continuing today, I have had a keen interest in a number of medical conditions including osteoporosis. In the early 1980's not much was known regarding this condition. As best exemplified by the *Brewer et al.* patent cited by the Examiner in the Official Action dated September 8, 1995, most of the emphasis at the time was on the N-terminal region, that could bind to certain receptor cells in bones. But the hPTH peptide was not well characterized and certain phenomena could not be explained by this binding. I and others sought to explain these hitherto unexplained phenomena. To test various theories, a good, inexpensive supply of very pure hPTH was needed. At the time, the only way to obtain hPTH was by extraction and isolation, followed by complex purification from human tissue. This was more than just a laborious process. Due to the difficulty in obtaining human tissue where hPTH had not deteriorated, relatively little material could be extracted and isolated at any one time.

Moreover, purification technology had not advanced to the point where suitable purity could be obtained.

4. Before I could continue my desired research, I first had to find or develop a suitable source of essentially pure hPTH. I felt that if suitable recombinant systems could be developed for producing hPTH, the inherent protein editing mechanism contained within the cell could be harnessed to produce, intact, correctly sequenced, fully active, hPTH. If this was accomplished, purification of the expressed protein could be carried out using the technologies that were prevalent at the time. I therefore sought the skills of my coinventors, and together we developed a source of raw material. The manner in which that was accomplished, and the resulting highly pure peptide, is described my above-captioned patent application.

5. By the use of recombinant technology as described in the patent application, we have been able to obtain hPTH which is not only of significantly higher purity than anything otherwise available, but also hPTH which was qualitatively superior. The data presented herein describing the attributes of the essentially pure, recombinant hPTH we developed are based on hPTH hormone produced by me or under my direct supervision in the mid to late 1980s. The resultant peptide was purified as described in the application. No other purification steps were employed.

6. Attached as Exhibits B-E are a number of glossy photographs labeled Glossy 0 through Glossy III. These glossies contain, among other things, photographs of electrophoresis gels run by me or under my direct supervision. Glossy 0 (Exhibit B) corresponds to an electrophoretic gel comparing synthetic hPTH obtained from the chemical supply company, Sigma, to recombinant hPTH obtained from yeast as disclosed in the above-captioned application. This gel was prepared before the filing of my patent application which issued as U.S. Patent No. 5,420,242. Lanes 1 and 4 contain chemical markers. Lane 2 (second from the left) contains recombinant hPTH prepared in accordance with the present invention. The Sigma material was loaded in Lane 3 (third from the left). The symmetrical blurring on either side of the actual hPTH band is the result of overloading the gel. It is significant to note that a single band of material is present in Lane 2 while three distinct bands are found in

Lane 3. This indicates the presence of significant quantities of high molecular weight impurity in the Sigma material.

7. Glossy I (Exhibit C) illustrates the gel of an experiment that was run on November 30, 1989 and shows a comparison screening of different chemically synthesized hPTH peptides from various companies including Peptide (Lanes 3 and 12), Peninsula (Lanes 4 and 13), Sigma (Lanes 5 and 14) and Bachem (Lanes 6, 7, 15 and 16). Two preparations of Bachem material were run (Lot Nos. ZE567 and 734B). Gels were loaded with either 200 nanograms or 800 nanograms of material as indicated, according to the manufacturers instructions. These materials were run against three lanes with molecular markers (Lanes 2, 11 and 20) as well as our recombinant hPTH produced from *E. coli* (Lanes 8 and 17), yeast (Lanes 9 and 18) and QPTH (Lanes 10 and 19). The materials obtained from Peptide and from Peninsula ran as a higher molecular species of much less quantity than indicated by the manufacturer and no correctly sized hPTH could be seen in the Peptide lanes, even when applied at 800 nanograms. The Peninsula material in Lane 4 shows a small indication of correct hPTH, but most of the material exists as a high molecular weight form. The Sigma preparation ran at a correct location but contained much less material than the manufacturer indicated. No material was evident at a loading of 200 nanograms. The two different batches from Bachem show a peptide of correct molecular size, but one of the preparations shows a heavy, and the other a lesser, trailing smear indicating lower molecular weight impurities. Again, the amounts of hTPH contained in the Bachem bands appear to be less than the amounts contained in the bands corresponding to the same loaded amount of recombinant hPTH from yeast and *E. coli*. Each of the three recombinant hPTHs appear as very sharp, fat bands, of equal intensity and much stronger intensity than any of the chemically synthesized preparations. When applied as 200 nanograms, only the recombinant hPTH lanes can be clearly seen. Everyone familiar with gel electrophoresis of protein knows that as little as 100 nanograms is usually sufficient to provide detectable staining. Thus, the absence of staining of 200 nanograms and diffuse bands at 800 nanograms are indicative of a relatively impure peptide.

8. Glossy II (Exhibit D) is a different experiment carried out on the same day as Glossy I. As indicated, two amounts of various materials, 200 nanograms and 800 nanograms, were applied per lane. The *E. coli* preparation used in this electrophoresis showed two high molecular weight bands on the silver nitrate stained gel (dimeric/polymeric aggregates) (Lanes 24 and 29), while the two yeast hPTH lanes contain very sharp bands in comparison to the Bachem preparation (compare Lanes 22 versus 23 and 27 versus 28, respectively). The Bachem preparation, shown in Lanes 23 and 28 shows considerable trailing toward degradation products. The amount of the correct material of the Bachem preparation may best be judged by the electrophoresis of the 200 nanogram sample. At 200 nanograms of material loaded, very little Bachem material was observed and lower molecular weight species are seen as a trailing area.

9. Glossy III (Exhibit E) is a blow-up of Glossy II indicating the size of the molecular markers in the first lane on the left. We have since carried out more recent electrophoresis and the bands appear exactly as they did in 1988 and 1989, indicating that there was no degradation of our preparation since its production in the late 1980's. The absence of degradation also indicates the substantial purity of the resulting material. The foregoing clearly indicates the superior purity of the material resulting in accordance with the present invention. However, it is also my opinion that because the hPTH produced in accordance with the present invention is recombinant material purified as explained in the application, not only is the peptide of better purity, but it is also of a significantly better quality. The differences are aptly illustrated in some of my prior published works.

10. For example, attached hereto as Exhibit F is a copy of my paper, "Differences in Binding Affinities of Human PTH(1-84) Do Not Alter Biological Potency: A Comparison Between Chemically Synthesized Hormone, Natural and Mutant Forms," published in the refereed journal, *Peptides*, (1994), 15, 1261-65. The data reported in this paper involved the analysis of hPTH material produced in accordance with the patent application in the mid to late 1980's. In fact, no other purification steps were taken, other than those disclosed in the patent application prior to the analyses described in this paper.

11. As will be readily apparent, we ran a number of *in vitro* tests to determine not only the purity, but also the qualities of the recombinant hPTH material prepared in accordance with the present invention and compared same to the best available chemically synthesized hPTH from Bachem. The first test, as illustrated in Fig. 1, shows the inhibition of radiolabeled [Tyr^{36}] chicken PTHrP (1-36) amide by various hPTHs. The data represents the mean (± standard deviation) of at least two independent experiments each performed in triplicate. As will be self evident from the figure, chemically synthesized hPTH had a calculated binding affinity, (K_d) of 18nM (95% confidence interval: 16.1-20.0nM) while recombinant hPTH (1-84) from both yeast and *E. coli* had a significantly lower apparent K_d of 9.5nM (95% confidence interval: 8.7-10.4nM).

12. Fig. 2 illustrates the stimulation of cAMP by different types of hPTH. The recombinant hormones in accordance with the present invention have the ability to stimulate intercellular cAMP accumulation with an EC_{50} of about 1.5nM, (95% confidence interval 1.0-2.2nM). In contrast, the solid phase synthesized hPTH showed a significantly reduced potency in stimulating cAMP production with an EC_{50} value of 5.7nM (95% confidence interval: 3.4-9.6nM). Fig. 2 also illustrates that the synthetically produced hPTH exhibited a reduced maximal response. Therefore, no matter how much synthetic hPTH is administered, it is not possible to obtain the same efficacy as that obtained by the administration of recombinant peptides in accordance with the present invention. These same results were mirrored in the *in vivo* testing undertaken and illustrated in Figs. 3 through 5.

13. A common way to measure hPTH bioactivity is to determine its ability to activate cell membrane-bound adenylate cyclase in target cells, e.g., bone derived cells. When hPTH binds to its receptor, adenylate cyclase is activated. This generates cAMP from ATP Mg. The activity of adenylate cyclase can be directly measured in membrane fractions of broken target cells when radioactive ATP Mg is added and the radioactively generated cAMP is isolated and quantitated by scintilation counting. The formation of cAMP

over the course of a certain time period represents a measure of adenylate cyclase activity. The hPTH produced as described in my patent application exhibits full activity in an adenylate cyclase assay. Therefore, the recombinant hPTH possesses biological activity which is substantially equivalent to that of naturally occurring hPTH. Another method of determining biological activity is to measure the amount of cAMP generated inside intact target cells which have been treated with hPTH in the presence of an inhibitor of cAMP degradation. After a certain period of treatment, the reaction is stopped (the cells are killed) and cAMP is measured after extraction by radioimmunoassay. This is the test described in my paper in the journal *Peptides* (Fig. 2). Thus, the adenylate cyclase and the cAMP assays, both individually and collectively, establish the full biologicol activity of the hPTH I and my coinventors made.

14. I have been warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon. I declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true.

Dated: 02.29.96

Kaare M. Gautvik

KAARE M. GAUTVIK, M.D.

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CURRICULUM VITAE

Kaare M. Gautvik, professor dr.med.

Personal and marital status

Name: Kaare M. Gautvik
Home address: Bregnevn. 3, 0875 Oslo, Norway
Business address: Institute of Medical Biochemistry, University of Oslo, P.O.Box 1112 Blindern, 0317 Oslo, Norway
Telephones: 47-22851055 (work); 47-22235137 (home)

Date and place of birth: 11th of December 1939 in Oslo.
Social Security No.: 111239.39311

Married to: Vigdis Teig Gautvik, date of birth: 24th of March 1947

Children: Lars Erlend Sakrisvold Gautvik, date of birth: 9th of January 1964
Silja Marie Sakrisvold Gautvik, date of birth: 31th of March, 1973
Ole Martin Teig Gautvik, date of birth: 21th of January 1982

Education

1. August 1958-June 64, Medical School at the University of Oslo.
2. 1967-69 Courses in mathematics involving geometry, statistics and mathematical analysis.
3. May 1970, Disputation for the medical doctor degree at the University of Oslo.
4. 1985, Specialist in medicine, in clinical chemistry and physiology.

Employment

1. June 1964 - June 1965, working at Tromsø University Hospital at medical and surgical departments.
2. July 1965 until December 1965, working as a general practitioner in Sjøvegan, Troms.
3. One year military service as a major in The Norwegian Air Force, working mainly at the Norwegian Institute for Aviation and Space Medicine.
   From 1967, position as post-doctoral researcher at The Institute of Physiology, University of Oslo.

5. From September 1969, promoted to Assistant Professor at the University of Oslo, Institute of Physiology.
6. Leader and responsible for clinical and experimental endocrinological laboratory of Institute for Surgical Res., The National Hospital, Oslo, from 1973 - 89.
7. From 1976-1978, training as a specialist in clinical chemistry at the Norwegian Radium Hospital, Oslo.
8. From August 1983 appointed to full professor at the Institute of Medical Biochemistry, Medical Faculty, University of Oslo.
   (At the same time receiving offers of professor chairs at the Institute of Physiology, Medical Faculty and at the Institute of Physiology and Biochemistry, Faculty of Odontology).

Post-doctoral training abroad

1. For three months in 1967, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.
2. From August 1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.
3. 1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.
4. 1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

Teaching responsibility

1. One year teching in aviation medicine for medical personnel and pilots.
2. I have given lectures and courses for medical students in following subjects: Haematology, kidney physiology, endocrinology, circulation, respiration and gastrointestinal physiology. From 1983 organized and given lectures and courses in molecular genetics at undergraduate and postgraduate level for students in medicine and sciences.
3. Organized interfaculty advanced courses within molecular endocrinology.
4. Lectures have been given in the following subjects at post-doctoral courses: Diseases of the thyroid gland (1973); Regulation of circulation in the gastrointestinal system (1973); Local hormones (1975); Endocrinology (annually from 1978); Tumour markers (1979); Calcium metabolism (annually from 1980); Ligands for peptide hormone-receptors, and Nuclein acid biochemistry (1984); TRH-receptors in prolactin-producing cells (1985). Molecular biology in medical research (yearly from 1983). Biochemical analysis on bone material (1991).
5. Invited lectures: Several places in the U.S., in Sweden, in Finland, and in England, as well as different places in Norway, a total of 37 as of 1995.
6. Chief organizer of post graduate scientific courses for the Medical Fakulty at University of Oslo, 1986-1991.

7. Organizer of international scientific meetings within the frame of the following societies: Acta Endocrinologica (European International Endocrine Society), The Scandinavian Physiology and Pharmacology Meetings, and the Norwegian Biochemical Society.
8. Introduced teaching in Molecular Biology for students at the Medical Faculty, Oslo.
9. Invited as Symposium Lecturer at international meetings in physiology and endocrinology and molecular biology as exemplified below:

Examples of specially invited symposium lectures

1. February, 1990: "Production of recombinant human parathyroid hormone in E.coli and Saccharomyces cerevisiae and its potential use as drug in osteoporosis" by Kaare M. Gautvik, Eli Lilly Co., Indianapolis, USA, in a Biotechnology meeting.

2. June, 1990: Symposium lecturer and organizer: "Hormone receptors and cellular signal transduction. The XXII Nordic Congress in Clinical Chemistry, Trondheim, Norway.

3. July, 1990: Symposium lecturer: "Transmembrane signal systems involved in the regulation of prolactin secretion by hypothalamic peptide hormones in cultured pituitary cells. 2nd European Congress of Endocrinology, Ljubljana, Yugoslavia.

4. July, 1990: Symposium lecture: "Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product". 5th European Congress on Biotechnology, Copenhagen, Denmark. (Unable to attend, and the lecture was held by cand.scient. Sjur Reppe).

5. August, 1990: Symposium lecture: "Processing and stability of human parathyroid hormone produced in E.coli and S.crevisiae studied by in vitro mutagenesis". Workshop/Symposium on site-directed mutagenesis and protein engineering, Tromsø, Norway.

6. December, 1990: Invited by Professor Guo Hui-Yu, Guangzhou, China and Professor G.L. French, Hong Kong. Lecture entitled: "Expression of human parathyroid hormone as a secretory protein in prokaryotic and eukaryotic microorganisms". The Second International Conference on Medical Microbiology and Biotechnology Towards 2000, Guangzhou, China. (Did not attend as a protest against the punishment of the students rebellion in Peking).

7. January 1991: Invited to a Workshop by Dr. Stephen Green, Central Toxicology Laboratory, ICI, Alderly Park, Maccelesfield SK10 4TJ,UK. Lecture entitled: "Synergistic effects of hormones and fatty acid on peroxisomal ß-oxydation, enzyme activities and mRNA levels".

8. January 1991: Invited to a Protein Engineering Meeting by Professor Ian Campbell, Biochemistry Department, Oxford University, Oxford, UK. Lecture entitled: "Cloning

and expression of human parathyroid hormone in microorganisms".

9. Invited by Professors T.T. Chen, D.A. Powers, B. Cavari, Maryland Biotechnology Institute, Baltimore, MD, to held a symposium lecture at the 2nd International Marine Biotechnology Conference, October 13-16, 1991, Baltimore, Maryland, USA. (Could not attend).

10. May 1991: Invited by Professor Jan Carlstedt-Duke, Karolinska Institutet, Huddinge, to held a lecture in the seminar series "Novum Lectures in Cellular and Molecular Biology".

11. January 1992: Invited by Professor Armen H. Tashjian, Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry on Molecular Pharmacology, Harvard Medical School, Boston, USA. Lecture entitled: "Use of antisense RNA in delineation of the mechanism of action of G-coupled hormones".

12. August 1993: Invited by Norwegian Society of Charted Engineers, The Blindern Conference. Lecture entitled: "Experience from industrializing basal research".

13. November 1993: Invited by Karolinska sjukhuset, Stockholm, to held a lecture at "Graduate course in molecular endocrinology - a problem oriented approach". The lecture is entitled: "Region specific actions of parathyroid hormone in target tissues".

14. February 1994: Invited by GBF, Gesellschaft für Biotechnologische Forschung mbH, Braunschweig. Lecture entitled: "Expression of human parathyroid hormone in microorganisms and animal cells with special reference to signal sequence efficacy and intracellular modifications".

15. September 1994: Invited by Professor K. Dharmalingam, Department of Biotechnology, Madurai Kamaraj University, India, to held a lecture in the symposium "Gene expression systems", XVIth IUBMB, New Delhi. Lecture entitled: "Expression of human parathyroid hormone in microorganisms, insect cells, mammalian cells and as a milk protein in transgenic mice".

16. November 1994: Invited by Professor A. Taschjian Jr., Harvard School og Public Health, Boston, to held a lecture in a seminar. Lecture entitled: "Certain structural and functional characteristics of the human TRH receptor cDNA and mapping of the gene".

17. February 11-13, 1995: Cairns, Australia, Workshop on "Animal Models in the Prevention and Treatment of Osteopenia"

18. February, 1995: Int. Meeting of Calcified Tissue Research, Melbourne, Australia.

Honorary lectures and prizes

1. In 1984 recipient of Professor Olav Torgersen's Prize and Memorial lecture. This prize and lecture was created by Professor Torgersen, the University of Oslo, who was one of the founders of the Society for Promotion of Cancer Research in Norway. Because he contributed with personal money, the prize and lecture had his name. The title of my lecture was: "The medullary thyroid carcinoma: a special type of familial and hormone producing cancer".
2. In 1984 I was given the international science prize called The Nordic Insulin Prize instituted by Professor Jacob E. Poulsen, who worked at the University of Copenhagen. This prize is given within endocrinology and the candidate is chosen from all the countries in Northern Europe. The money was donated by the Insulin Laboratory now the company Novo-Nordisk. At that time, only one Norwegian had previously received this prize. The prize was given for my studies regarding how hormones exerted their biological actions in target cells.
3. The Gunnerus Prize was given in 1986 by the Royal Society of Norwegian Scientists. This is a prize which is given to a scientist selected by this society for scientific merits obtained and again it was within the field of hormone structure and action.
4. In 1987 I received a prize within biotechnology created by the Research Park at the University of Oslo, which at that time was called the Innovation Centre, University of Oslo.
5. Novum Lectures in Cellular and Molecular Biology, which was associated with a scientific prize. Invited by Professor Jan-Åke Gustafsson at Novum, Huddinge, The Karolinska Institute, Sweden, in 1991. This was given based on my research with human parathyroid hormone in relation to its first cloning, expression and studies of actions.
6. Lectures at Harvard School of Public Health in Cellular and Molecular Biology in 1995, regarding cloning of hormone genes and their characterizations. Invited by Professor A.H. Tashjian Jr. at the Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, USA.

Referee activity

I am or have been working as referee for the following international journals:

Endocrinology
J. Expl. Cell Res.
Acta Physiol. Scand. (Kbh.)
Eur. J. Endocrinol. (Acta Endocrinol. Scand. (Kbh.)
Eur. J. Clin. Invest.
Hormone Research
Acta Obstet. Gynecol. Scand.
Journal of Endocrinological Investigation
Eur. J. Biochem.
Experimental Cell Research
Scand. J. Gastroenterol.

Guidance for the academic doctor degree

1. Veterinary, dr.lic. Richard Tollman: "Parturition hypocalcaemia in cows". 1976. Oslo.
2. Dr.med. Trine Normann: "Medullary carcinoma of the thyroid. A morphological, clinical and experimental study". 1977. Oslo.
3. Dr.med. Egil Haug: "Prolactin and growth hormone secretion by rat pituitary cells in culture. Hormonal control and mechanism of action". 1978. Oslo.
4. Dr.med. Bjørn Klevmark: "Motility of the urinary bladder in cats during filling at physiological rates." Oslo. 1978.
5. Dr.odont. Torill Berg Ørstavik: "Glandular kallikreins. Origin and secretion in some exocrine organs of the rat". 1978. Oslo.
6. Dr.med. Arne Ekeland: "The role of calcitonin in fracture healing". 1981. Oslo.
7. Dr.philos. Kjersti Sletholt: "Calmodulin from rat anterior pituitary tumour cells and its biological significance". 1988. Oslo.
8. Dr.med. Trine Bjøro: "Regulation of prolactin secretion by hypothalamic hormone with special emphasize on vasoactive intestinal polypeptide (VIP)". 1988. Oslo.
9. Dr.scient. Øyvind Andersen: "Purification and characterization of salmonid prolactin". 1989. Oslo.
10. Dr.scient. Marianne Wright: "Biochemical studies of the pituitary receptor for thyrothropin-releasing hormone. Cell surface receptor protein characterization, receptor mRNA isolation and cDNA library generation and screening". 1991. Oslo.
    Dr.phil. Vendela Parrow: Signal transduction and gene regulation in cultured endocrine cells. 1991. Oslo.
11. Dr.med. Eyvind J. Paulssen: "G protein-coupled transmembrane signalling in prolactin-producing rat pituitary tumour cells". 1992. Oslo.
12. Dr.philos. Ruth H. Paulssen: "G protein-coupled transmembrane signalling in prolactin-producing rat pituitary tumour cells". 1992. Oslo.
13. Dr.scient. Hilde Nebb Sørensen: "Actions of hormones and fatty acids on peroxisomal ß-oxidation enzyme activities and gene transcription." (Disputation 1993).
14. Dr.philos. Najma Kareem: "The use of protein engineering to study hormone processing and secretion in different host cell systems". (Disputation 1994).
15. Dr.philos. Ole Kristoffer Olstad: "Expression, purification and characterization of recombinant parathyroid hormone like peptides". (Disputation 1995).
16. Dr.med. Berit Mortensen: "The influence of vit. D_3 on bone remodelling: In vitro and in vivo studies of bone turnover in the normal and uraemic conditions". (Disputation 1995).
17. Dr.philos. Venke Skibeli: "Structural and functional aspects of Atlantic salmon growth hormone and prolactin". (Disputation 1996).
18. Dr.med. Erik Rokkones: "Expression of heterologous peptide hormone genes in cultured cells and in animals". (Disputation 1996).
19. Dr.philos. Sjur Reppe: "Secretion of heterologous proteins from the yeast Saccharomyces cerevisiae". (Disputation 1996).

Supervision of postgraduate candidates, thesis works:

I. Cand.pharm. Per Wiik Johansen: "Regulation of prolactin and growth hormone secretion and synthesis by bromocriptine in rat anterior pituitary tumour cells". (Disputation 1996).
II. Cand.scient. Vilborg Matre: "Cloning and expresjon of membrane receptors for hypothalamic hormones in exitable cells".
III. Cand.scient. Hilde Hermansen Steineger: "Studies of regulatory gene-elements and transcription factores that mediate peroxisomal inducton and proliferation".
IV. Cand.scient. Ole Petter Løseth: "Studies on hormonel bone remodulation in tissues and in animals".
V. Cand.scient. Per Ivar Høvring: "Structure analysis and functional studies of cloned thyroliberin receptor and receptor isotypes".
VI. Siv.ing. Edith Rian: "Expression of parathormone-like peptides in tumour cells".

Supervision of students' main degrees

1. Cand.pharm. Åse Aulie: "The effect of somatostatin on cultures of growth hormone and prolactin producing cells." University of Oslo. 1979.
2. Cand.pharm. Per Wiik Johansen: "The effects of bromocriptin on prolactin and growth hormone producing rat pituitary gland cells in culture." University of Oslo. 1981.
3. Cand.pharm. Nina Lillegraven: "The significance of extracellular ion influence on the binding of thyroliberin to rat pituitary gland cells in culture." University of Oslo. 1982.
4. Stud.med. Eyvind J. Paulssen: "The effect of TRH and oestradiol on prolactin-synthesis in rat pituitary cells in culture." University of Oslo. 1983.
5. Cand.pharm. Kari Furu and cand.pharm. Kirsten Kilvik: "The uptake mechanism for oestradiol in rat pituitary cells in culture." University of Oslo. 1984.
6. Cand.pharm. Berit Taranrød Johansen: "Cloning of mRNA for rat prolactin". U niversity of Oslo. 1986.
7. Cand.scient. Marianne Wright: "Characterization of surface proteins of GH-cells with special reference to the TRH receptor". University of Oslo. 1987.
8. Cand.scient. Jenny Owe: "Binding and degradation of thyrotropin releasing hormone in hormone producing rat at pituitary cells in culture". University of Oslo. 1988.
9. Cand.real. Grete Sørnes: "Effects of vitamin D on $^{45}Ca^{2+}$ efflux and prolactin production". University of Oslo, 1988.
10. Cand.pharm. Siv Eriksen: "Development of an in solution mRNA hybridization test using antisense mRNA probes for prolactin". University of Oslo. 1988.
11. Cand.real. Tom Skyrud: "Effects of human growth hormone and IGF-I on growth and clinical chemical plasma parameters". University of Oslo. 1988.
12. Cand.pharm. Laila Norrheim: "The inductive effect of tetradecyl-thio-acetic acid on peroxisomal ß-oxidation in 7800 C1 Morris hepatoma cells is stimulated by dexamethasone and inhibited by insulin". University of Oslo. 1988.
13. Dipl.ing. Kristin Austlid Taskén: Transfeksjonsstudier i Karpe- og rotte hypofyseceller. University of Trondheim (NTH)/University of Oslo. 1988
14. Cand.real. Hilde Nebb Sørensen: "The mechanism of ^{3}H-Dexamethasoneuptake into

7800C_1 hepatoma cells in culture". University of Oslo. 1989.

15. Stud.real. Najma Kareem: "Secretion and processing of recombinant hPTH in E.coli. Significance of preprosequences". University of Oslo. 1989.
16. Stud.ing. Edith Rian: "Expression of parathormone-like peptides in tumour cells.
17. Cand.scient. Even Sollie: "Stability of peroxisomal ß-oxidation enzyme activity and mRNA levels". University of Oslo. 1993.
18. Stud.scient Åse-Karine Fjelheim: Cloning and characterization of the human thyrotropin releasing hormone. (1996).

Guest research workers from abroad

In my group we have had research visitors for periods of one to three years from Polen, Bulgaria, Sweden, Tyskland, Denmark, Iceland, India, Israel and USA.

Member of comittees for the academic doctor degree in Norway and abroad.

1. Opponent at dr.med. Bjørn Biber's disputation at Physiological Institute, The University of Gothenburg, Sweden. The work represented gastrointestinal physiology. 1974.
2. First opponent at dr.med.vet. Knut Hove's disputation. The work represented the effect of insulin on the intermediate metabolism in ruminants and mammary gland physiology. University of Tromsø, Norway, 1978.
3. First opponent at siv.ing. Kirsten Sandvig's disputation on the work: "Interaction of the toxic lectins abrin, ricin, and modeccin with mammalian cells". The work includes biochemical examinations on absorbtion and effect of toxic lectins in cell cultures. University of Oslo, Norway, 1979.
4. Opponent at cand.real. Anne Sundby's disputation on the work: "Plasma testosterone in young bulls in relation to age, gonadotropin stimulation and rate of weight gain and some studies on testicular gonadotropin receptors". University of Oslo, Norway, 1982.
5. Opponent at siv.ing. Anders Sundan's disputation on the work: "Studies on the entry of modeccin, diphtheria toxin, ricin, and pseudomonas toxin into mammalian cells". University of Oslo, Norway, 1985.
6. Opponent at dr.med. Svein Dueland's disputation on the work: "Absorption and transport of vitamin D_3 and 25-hydroxy-vitamin D_3 in the rat". University of Oslo, Norway, 1986.
7. Opponent at dr.philos. Dagny Sandnes's disputation on the work: "Beta-adrenoceptors on rat hepatocytes and human mononuclear leucocytes, with special reference to quantitation and regulation". University of Oslo, Norway, 1988.
8. Opponent at dr.med. Øyvind Sverre Bruland's disputation on the work: "Preparation and properties of two novel highly specific antisarcoma monoclonal antibodies and their application in the characterization and diagnosis of human sarcomas". University of Oslo, Norway, 1989
9. Opponent at dr.med. Pål Wiik's disputation on the work: "Vasoactive intestinal peptide as a modulator in the neuro-immune axis; the influence of stress". Norwegian Defence Research Establishment, Norway, 1989.

10. Opponent at dr.med. Eystein S. Husebye's disputation on the work: "Stimulus-secretion coupling in chromaffin cells of the bovine adrenal medulla. With special reference to the role of phospholipid metabolism". University of Bergen, Norway, 1990.
11. Opponent at dr.scient. Hooshang Lahooti's disputation of the work: "The estradiol receptor and the 90 kDa heat shock protein. Phosphorylation of the receptor and the heat shock protein, and studies on regulation of the estradiol receptor mRNA". University of Bergen, Norway, 1991.
12. Leading the disputation of Hilde Nebb Sørensen on the work: "Hormonal modulations of fatty acid stimulated peroxisomal ß-oxidation in cultured liver cells." University of Oslo, Norway, 1993.
13. Opponent at dr.odont. Janicke Liaaen Jensen's disputation on the work: "Human saliva: Biochemical and physiological aspects of some components", Faculty of Density, University of Oslo, Norway, 1994.

Member of advisory international/national committies for evaluation of professor positions.

1. Professor Ingrid U. Richardson, Harvard University, Boston, USA. 1974.
2. Professor Thomas F.J. Martin, University of Wisconsin, USA. 1984.
3. Professor Margaret A. Brostrøm, University of Medicine and Dentistry of New Jersey, USA. 1984.
4. Position as full Professor at the Institute of Physiolology, University of Gothenburg, Sweden. 1987.
5. Professor I at the Department of Clinical Chemistry, University of Tromsø, Norway, 1987.
6. Participation in an international board created by Sandoz, Basel, Switzerland, for nominating a candicate for the Sandoz International Endocrinological Prize in 1988.
7. Professor in Veterinary medicine, Norwegian Veterinary University, Oslo, Norway, 1989.
8. Position as Professor in Physiological Chemistry, University of Kuopio, Finland. 1989.
9. Position as Professor I in Endocrinology at the University of Gothenburg, Sweden, 1991.
10 Position as Associate Professor I in Endocrinology, University of Gothenburg, Sweden, 1993.
11. Position as Associate Professor I in Biochemistry, University of Bergen, Norway, 1993.
12. Appointed member of committy to evaluate chair Professorship at Karolinska Institution, Dept. of Endocrinology, Sweden (I had to decline because of sabattical year).

Honorary Societies

Member of the Norwegian National Academy of Science and Letters

Professional memberships

Norwegian Society of Biochemistry
Norwegian Society of Physiology
Norwegian Society of Endocrinology
Endocrine Society (USA)
Society for Calcified tissue (USA)
Society for Bone and Mineral Metabolism (USA)

Medical clinical specialities

1. Clinical Physiology and Chemistry including Nuclear Medicine
2. Work Medicine

Medical Faculty Responsibilities

1. An elected member of the Medical Faculty 1987-1990.
2. A member of the Reesearch Council at the Medical Faculty 1987-1990.
3. Chairman of Postgraduate Courses for Ph.D. and Dr.med. students at the Medical Faculty 1986-1991.
4. Member of the Institute Group Committee for the Preclinical Sciences from 1989 and present.
5. Member of the Medical Faculty's councel for evaluation of postgraduate applications from 1989-1993.

Research Council Responsibilities

1. Chairman for the Biotechnology Committee as a representative for Norwegian Research Council in an inter research council body, 1986-1989.
2. Member of The Norwegian Research Council for Science and the Humanities (NAVF) Committee for Physiology and Pharmacology, 1986-1989.
3. Development and function as responsible leader of the nationwide core facility for peptide synthesis, 1988-1991.
4. Member of the International Scientific Board of Novo-Nordisk Research Committee.
5. Member of the CIBA Foundation Scientific Advisory Panel from 1995 elected as representative from Norway.

Patents

General information

Two U.S. patents, U.S. Patent No. 5.010 010 and No. 5.420.242 are held with

international extensions in Europe, Japan, Canada, and Australia. In addition, three Divitional Applications are submitted to the U.S. Patent Office and elsewhere.

All of Gautvik et al.'s patents and patent applications in the different countries are covering specific methods related to the production, purification and characterization of PTH in microorganisms.

A BRIEF DESCRIPTION OF THE MAIN RESEARCH PROJECTS GIVEN IN HISTORICAL ORDER:

I. THE BIOCHEMICAL AND PHYSIOLOGICAL STUDIES RELATED TO PLASMA KININS AND KALLIKREINS

II. MOLECULAR ENDOCRINOLOGICAL RESEARCH

- A. REGULATION OF HORMONE SECRETION AND SYNTHESIS
- B. RECEPTOR FUNCTION AND CLONING OF NEUROENDOCRINE HORMONE RECEPTORS
- C. STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

III. ENDOCRINOLOGICAL RESEARCH RELATED TO HYPERFUNCTION OF THE PARATHYROID GLAND AND RESEARCH IN RELATION TO MEDULLARY CARCINOMA OF THE THYROID GLAND

- A. CLONING AND EXPRESSION OF PARATHYROID HORMONE AND RELATED PEPTIDES IN MICROORGANISMS, MAMMILIAN CELLS AND TRANSGENIC ANIMALS
- B. PARATHYROID HORMONE RELATED PEPTIDE AND MALIGNENT HUMORAL HYPERCALCAEMIA
- C. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE OSTEOSARCOMA PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION
- D. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS

IV. MOLECULAR ENDOCRINOLOGY STUDIES IN FISH

- A. STUDIES OF GENE EXPRESSION IN TRANSGENIC FISH
- B. ISOLATION, PURIFICATION, AND CHARACTERIZATION OF S ALMON PROLACTIN AND GROWTH HORMONE

I. THE BIOCHEMICAL AND PHYSIOLOGICAL STUDIES RELATED TO PLASMA KININS AND KALLIKREINS

Plasma kinins are biologically very active polypeptides that are distributed throughout the body and become accepted to be of importance for regulation of blood flow in certain organs. Kinin forming enzymes (kallikreins) and kinin inactivating enzymes (peptidases, "converting enzyme") have received new attention the last years due to their possible involvement in hypertension. My thesis from April 1970 dealt with certain physiological/biochemical aspects in relation to blood flow regulation including purification of substrates for kallikreins and their characterization in vitro. These components were used for studying their physiological interaction during perfusions of the salivary gland activated via nerve stimulation. By using purified substrates and their enzymes, a direct functional involvement of plasma kinins in functional vasodilatation could be demonstrated in vivo for the first time. Its title was "Studies on vasodilator mechanisms in the submandibular salivary gland in cats" (O.A.: 7).
Relevant references: R/C, 3,6,7,8,11,12,13; O.A, 1-6,8,9,11,16,19,23, 39,43,50, 55,56.

II. MOLECULAR ENDOCRINOLOGICAL RESEARCH

A. REGULATION OF HORMONE SECRETION AND SYNTHESIS

A major part of my research engagement has been carried out using functional cell cultures and transplantable tumors from highly differentiated cells that are able to perform organ specific functions. The following areas have been actively persued since 1971:

i. The biological effects and mechanism of actions of the hypothalamic hormones thyroliberin, dopamine, somatostatin and vasoactive intestinal polypeptide in prolactin and growth hormone producing rat pituitary cells.

These hypothalamic hormones are of central importance in regulation of release of prolactin and growth hormone from the anterior pituitary gland. The results which we have obtained with the cultured rat pituitary cells, have all been confirmed in more physiological endocrine model systems and thus appear to be valid for interpretation of how these regulatory hormones influence the functions of the anterior pituitary gland. I have carried out characterization of receptor binding of thyroliberin and been a senior researcher to originate research regarding receptor characterization for dopamine, somatostatin and vasointestinal polypeptide. In addition, I have steadily persued studies to elucidate and delineate the mechanisms of action for these peptide hormones. We have characterized the second messengers systems involving cyclic nucleotides and calcium, as well as described pathways of phospholipase C activation with formation of inositol triphosphates and

diacylglycerol. I have been one of the first in this research area to show the involvement of cyclic nucleotides and calcium in the action of thyroliberin, dopamine, somatostatin and vasoactive intestinal polypeptide. How these second messengers were generated and their interaction, were first described in an invited review article for the Benzon Symposium, Copenhagen, 1988. (K.M. Gautvik et al., Regulation of prolactin secretion and synthesis by peptide hormones in cultured rat pituitary cells, Alfred Benzon Symposium 25, Copenhagen, 1988) and as an invited lecture at the 2nd European Congress in Ljubljana, 1990.

The ongoing research has concentrated on the involvement of GTP binding proteins in the receptor coupling of these hormonal signals as well as the characterization of the receptor itself. Thus we have identified and studied the functional coupling between these receptors and the G protein subunits in pituitary cells. Furthermore, these studies are now completed with a description of the adenylyl cyclase subclasses in the same cell-types and their engagement by the different hormone-receptor G protein subunits. These studies have involved measurements of specific mRNAs, the corresponding proteins and their regulation by the hypothalamic hormones as well as antisense RNA experiments testing the direct physiological involvement of G_{α_s} protein in the action mechanism of e.g. thyreoliberin.

ii) <u>The biological effects and mechanisms of action of steroid hormones (oestradiol, progesterone, testosterone, cortisone and vitamin D_3) examined in prolactin and growth hormone producing cells in culture</u>.

Through the years 1973-1983, I was engaged in studying the effects of the above mentioned steroid hormones and characterization of their distinct receptors in prolactin and growth hormone producing cells in culture. We showed e.g. for the first time the existence of testosterone and vitamin D_3 receptors in adenopituitary cells. The biological effects of these hormones and how they regulate prolactin and growth hormone synthesis, were also examined in detail. How the steroid and polypeptide hormones regulated hormone receptor levels was studied during different conditions with the aim to understand their physiological interplay.

iii) <u>Regulation of rat prolactin and growth hormone gene expression in functional pituitary cells</u>.

Many of the hormones which affects prolactin and growth hormone secretion are also able to change the rate of synthesis for these hormones. In the same decade, we developed immunopresipitation methods for the radioactively labelled hormones, and improved the sensitivity of hormone measurements to the level of single cells. This was shown using capillary tube gel electrophoresis of immunoprecipitated hormones and the results confirmed by concommitant immunocytochemistry. By using a combination of protein analysis and RNA blotting methods, we could show that prolactin synthesis was stimulated mostly by thyroliberin and oestrodiol while an inhibitory effect was found by dopamine and cortisone. The most efficient inducer of growth hormone synthesis was cortisone, and its

synthesis was also inhibited by dopamine.
Relevant references: O.A.: 12-14,22,24,25,27,31-34,36-38,42,44,48,49,51,57-59,62,66, 67,69,71-73,76,77,80,81,83-85,87,91,92,96-105,107,109,110,112,114,115,122,132, 133,134; R/C: 17,19,21,22,23,26,28-30,34.

B. RECEPTOR FUNCTION AND CLONING OF NEUROENDOCRINE HORMONE RECEPTORS

In this respect, we have isolated and cloned cDNA for the receptor for thyroliberin in rats and in humans, the latter result as the first original description in the litterature. Prior to this work we characterized and visualized this receptor both by photo affinity labelling using the radioactive hormone as well as using a polyclonal antiserum made by us and raised against cell surface ideotypes. We cloned the rat and human receptor after making a mRNA based PCR product and a cDNA which was then used as probe for screening libriaries.

The human thyroliberin receptor, shows several interesting features when the aminoacid sequences are compared in rat and human. The differences have probably direct bearing on the functional activity of the receptors in relation to G protein coupling pattern as well as signal effector activation, which we in the rat have described in detail.

Relevant reference: O.A. 115,130,157.

C. STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

The hypothalamus consists of discrete nuclei which paly a vital role in several biological functions that are essential to mammals and related to different homeostatic mechanisms, reproduction, behavior, emotion and responses to various stress. The hypothalamic nuclei in part, integrate many autonomic regulatory systems whose final path is expressed by neuroendocrine cells. Thus, they represent "high command centers" within the endocrine system and enable the central nervous system to initiate, adjust and balance intricate and complex endocrinological reflexes. This part of hypothalamic function is exerted by synthesis and release into the pituitary portal system of a number of substances, mainly of peptide and amine nature. These substances control the anterior pituitary gland function. In addition, hypothalamus is the site of production of two hormones, oxytocin and vasopressin, which are transported by axon flow into the posterior pituitary from which they are released into the general circulation affecting salt/water balance and being of

importance for parturition and breast feeding. Furthermore, the hypothalamus is a center for social and sexual biological behaviors and mediates feeding and drinking habits. The importance of hypothalamic functions in the field of calorie balance may be exemplified by the recent discovery by Zhang et al. (Nature, 372, 1994) who describe the first cloning of the so called mouse "obese" gene and its human analog, where the receptor for this hormone by all criteria has to be localized in a hypothalamic nucleus. Since the first objective of this research has been obtained, namely to generate a subtracted hypothalamic specific cDNA library of high quality, we will expect to find this receptor among the 10^5 independent clones present in the library, as well as clones of mRNA encoding a large number of other novel proteins.

The subtraction library shows inserts with sizes estimated on agarose gel electrophoresis between 0.4 and 1.2 kb (mean > 0.7 kb), a result which was very satisfactory.

The quality of the library is further assessed by the extent to which clones of certain mRNAs known to be present in hypothalamus had been amplified during subtraction, and to what extent clones of mRNAs which are ubiquitously present in the central nervous system had been removed. Vasopressin which is exclusively present in hypothalamus is enriched 20-30 times in the subtracted library compared to the hypothalamus cDNA library, and the commonly present NSE and cyclophilin is completely removed after subtraction. 215 clones from the subtracted library have been picked into grids and hybridized with probes prepared by PCR amplification of the inserts from the driver, target and subtracted libraries. Approximately 1/4 of the clones give substantially greater signals with the subtracted target probe than the unsubtracted target probes and faint or undetectable signals with the driver probe. If validated, these figures suggest that roughly 1% of the hypothalamus mRNA mass is enriched in that structure (corresponding to an estimated 300 different gene species, given that 30,000 species are expressed in the brain).

Conclusion: The results so far from our learning and usage of a powerful and highly sensitive novel subtractive nucleic acid hybridization method are summarized. The generated hypothalamic subtraction library appears to give a specific and comprehensive representation of mRNAs that are not present in other brain areas as hippocampus and cerebellum. One article on the general aspects of hypothalamic enriched/specific mRNAs is in preparation. Another article is describing a novel somatostatin-like peptide, called cortistatin. This article is in press 1996 in Nature.

III. ENDOCRINOLOGICAL RESEARCH RELATED TO HYPERFUNCTION OF THE PARATHYROID GLAND AND RESEARCH IN RELATION TO MEDULLARY CARCINOMA OF THE THYROID GLAND

This research started out in the early 70'ies as a result of my development of two radio-immunoassays for parathyroid hormone and calcitonin, methods which at that time did not exist in Norway. Since 1973, I have thus carried out clinical laboratory diagnostic activity

for the whole country, and also received samples from other Scandinavian countries as well as England. In different collaborative studies, we used these assays in basal and clinical endocrinological research related to how these hormones were regulated by calcium both in vitro and in vivo. In addition, biochemical work was carried out in order to characterize different intracellular hormone-forms retrieved from tumor cells producing these peptides.

The hyperfunction of the parathyroid glands occurs in relation to development of adenomas and/or hyperplasia. The cause(s) of primary hyperparathyroidism is (are) unknown, while secondary hyperparathyroidism occurs as a result of chronically lowered serum Ca^{2+} (e.g. in chronic renal failure). The question about how low concentration of serum Ca^{2+} may induce not only increased hormone secretion and synthesis, but also trigger DNA replication and cell proliferation, is intriguing, but still unknown. We have studied patients with secondary hyperparathyroidism during various experimental conditions to address these questions. From human adenomas we isolated poly(A)$^+$ RNA for cloning of parathyroid hormone (PTH) in 1983.

Medullary carcinoma of the thyroid gland (MCT) occurs as an inherited and spontaneous malignant disease. As the first to introduce diagnostic tool to discover this calcitonin producing tumor in Norway, we have mapped the extent of the disease in our country.

Relevant references: O.A.: 28-30,40,41,52-54,61,65,68,70,74,78,79,82,86, 90,94,108, 117; R/C: 5,15,16.

A. CLONING AND EXPRESSION OF PARATHYROID HORMONE AND RELATED PEPTIDES IN MICROORGANISMS, MAMMILIAN CELLS AND TRANSGENIC ANIMALS

This project started in 1983 and developed into a major research engagement where we have succeeded as the first in the world, to clone and express this hormone in 100 mg quantities both as a product in E.coli as well as in Saccharomyces cerevisia. We have also successfully transfected mammalian cell cultures, insect cells and transgenic animals, silkworm larvae and mice, and obtained expression of this hormone. We have also expressed mutated forms in yeast and studied the intact hormone and fragments in insect cells and Bombyx mori larvae with the aim to understand cellular processing, trafficking and secretion.

In the first part of the work we cloned the cDNA for human parathyroid hormone using conventional cloning techniques and expressed the peptide as met-gly product in E.coli. This peptide analogue was not biological active. In a search for peptides with antagonistic action, we found that gly PTH (1-84) was an interesting form having binding properties and inhibited the agonist by 40 per cent at 10^{-9}M. In clinical medicine an effective PTH antagonist will be of potential use both as a diagnosticum and in treatment of hypercalcaemia.

For expression in S.cerevisiae recloning of the cDNA was carried out using a fusion construction with the prepro region of the yeast mating factor α gene. We were able to obtain an effective and correct N-terminal processing and isolated human PTH as a secretory product in a yield (up to 10 mg/l). We also developed a down-stream technology for purifying the hormone which was shown to be identical to the authenthic peptide hormone by a variety of chemical, biochemical and biological test systems.

In order to obtain a higher yield of human parathyroid hormone, we in vitro mutagenized a proteolytic cleavage site internal to the peptide, and obtained a full-length agonist (84 amino acids) which after purification was shown to have the same biological activity as the authentic hormone.

A part of this work has been concentrated on to find optimal signal sequences both for expression in E.coli as well as in yeast, where we by using different amino acid substitution in new gene constructs, have developed an effective test system for looking at N-terminal processing. This is obtained by making a fusion gene between the N-terminal region of the PTH gene and the protein A gene which in E.coli is transcribed using the protein A promoter and transcription stop signals.

We have also made constructs for use in mammalian cells where we employ the Whey Acidic Protein (WAP) promoter region in order to express PTH in mammalian cells of mouse origin. We have recently in addition generated transgenic mice who express PTH as a secretory product in milk.
Relevant references: O.A.: 124,128,129,133,139,140,144,145,161,162,163,166, 167,168; R/C: 31,32,35.

B. PARATHYROID HORMONE RELATED PEPTIDE AND MALIGNENT HUMORAL HYPERCALCAEMIA

Parathyroid hormone related peptide has been isolated as the causative agent during conditions of malignant humoral hypercalcaemia. This condition occurs in the presence of several malignent diseases such as cancers, carcinomas as well as myelomatosis where the tumour cells are able to produce a parathyroid hormone related peptide. We have received cDNA clones and recloned it in yeast for production of the protein to be used in receptor binding- and activation-studies. We are also studying the expression of this gene in animal and human tumour cells, with the purpose to learn gene specific splicing.
Relevant references: O.A.: 151; 160, Jemtland et al., Rian et al. submitted; R/C: 36.

C. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE OSTEOSARCOMA PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION

By using the same novel subtractive hybridization procedure as employed and described in

Chapter II,C, we have generated a subtracted cDNA library using the osteosarcoma phenotype cDNA library as made from three different human osteosarcoma cells from which is subtracted the cDNA library obtained from normal human osteoblasts. The subtraction is performed by using cDNA from osteosarcoma cells minus RNA transcribed from the corresponding cDNA library of the normal osteoblast. These are experiments in progress and we are about to describe individual cones obtained from a subtracted library of about 400.000 independent colonies. The aim of this study is to identify those mRNAs which are overexpressed or lacking in the osteosarcoma phenotype and compile these results in order to have a greater understanding regarding how a normal cell is transformed into this tumortype.

D. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS

Parathyroid hormone is the most important physiological regulator of bone formation. This hormone therefore is assumed to represent an important drug in the prevention and especially treatment of postmenopausal osteoporosis. However, as a succession of our previous work regarding the studies of this hormone, we have continued to search for a complete overview of all gene products that parathyroid hormone is stimulating in bone cells in order to isolate the mRNAs and corresponding proteins which may be of sentral importance for the development of osteoporosis - or which may be called "the genes for osteoporosis". Again by using the same molecular substraction method as described in Chapter II, C, we use this time parathyroid stimulated normal bone cells cDNA library minus RNA transcribed from generated libraries of normal bone cells. This work is in its initial phases.

IV. MOLECULAR ENDOCRINOLOGY STUDIES IN FISH

A. STUDIES OF GENE EXPRESSION IN TRANSGENIC FISH

The endocrinological aspects as it relates to regulation of growth and development of fertility, are as important in fish as in mammalian species. In addition, fish is an i nteresting model system also for studies within embryology, differentiation, and gene regulation. As in mammalian species, growth hormone will regulate growth and prolactin will be of importance for normal fertility and adaptation to salt/fresh water conditions. Also, in this research area, we have worked partly from the protein side and partly from the DNA side. We have as first reports described isolation and purification of prolactin and growth hormone from Atlantic salmon, and developed sensitive radioimmunoassays in order to follow the hormones in fish as a function of age and also during different experimental conditions.

The initial DNA work for production of transgenic fish is published and may be summarized briefly:

We first developed a new microinjection technique where small amount of foreign DNA was injected into fertilized fish eggs and the survival rate was more than 90%.

As the first model gene we used the human growth hormone gene (kindly given from Professor R. Palmiter, USA) where the promoter for the metallothionin gene ensured expression in eukaryotic organisms. From this gene construction, we made cDNA probes which specifically hybridized to growth hormone DNA and mRNA.

The microinjected DNA for human growth hormone gene incorporated in the embryo's chromosomal DNA was isolated and demonstrated by Southern blot analysis. It was incorporated in the fish genome already after 7 days.

We also showed that the human growth hormone gene was active based on the occurrence of specific mRNA for human growth hormone and production of growth hormone by the fish embryo and secretion to the medium. This achievement shows that it is possible to develop a rather unique model to study gene expression both under embryonic development and in the adult fish (R/C 44; O.A. 119, Skibeli submitted 1996).

In order to measure expression of Atlantic salmon growth hormone, we have isolated and purified this hormone as well as prolactin from the same species. (O.A. 118, 121, 135, 138).

B. ISOLATION, PURIFICATION AND CHARACTERIZATION OF SALMON PROLACTIN AND GROWTH HORMONE

At the time we started out this project, a preliminary sequence of the corresponding hormones in the Pacific salmon was known. During this project we were however, able to purify and characterize both these hormones from the Atlantic salmon and were the first to give the amino acid sequence data on both these hormones. In addition, we used our own made prolactin antisera for studying the possible function and involvement of prolactin in sex maturation of Atlantic salmon. Growth hormone was further characterized by detailed chemical and biochemical analysis including phosphorylation and glycylation patterns, development of antisera against fragments of the hormone, and analysing the immunoreactivity of growth hormone from different salmon species. These reports describes for the first time GH species as two gene products in Salmon fish, and they are both glycoproteins, and one also phosphoglycoprotein.

Relevant references: R/C 44; O.A. 118, 119, 135, Skibeli submitted 1996).

THE MAIN RESEARCH ACTIVITIES DURING THE LAST 4 YEARS AND FUTURE SCIENTIFIC ENGAGEMENT:

I. PARATHYROID HORMONE (PTH) AND PARATHYROID HORMONE RELATED PROTEIN (PTHrP)

The aim for this work was to produce:

i) Recombinant parathyroid hormone for structure activity studies in relation to bone cell activation.
ii) Study intracellular processing and trafficking of these hormones and to compare signal sequence efficacy in different host expression systems.

We were the first in the world to clone and produce full-length human recombinant parathyroid hormone in mg quantities. For this work we developed gene constructs, vector modifications, fermentation technological improvements as well as complete methods for down-stream technology. The final product is PTH identical and more than 99% pure and has shown full chemical, biochemical and biological identity with the intact hormone. These results are written in the following articles that are printed.

We have also been as indicated by the list of references below, the first in the world to express secreted human parathyroid hormone in mammalian cells as well as a secretory milk product in transgenic mice. In addition, we have been the first to develop full-length PTH polypeptides with agonist and antagonist functions.

1. Høgset A, Blingsmo OR, Gautvik VT, Sæther O, Jacobsen PB, Gordeladze JO, Alestrøm P, Gautvik KM. Expression of human parathyroid hormone in Escherichia coli. BBRC, 166:50-60, 1990.
2. Gabrielsen OS, Reppe S, Sletten K, Øyen TB, Sæther O, Høgset A, Blingsmo OR, Gautvik VT, Gordeladze JO, Alestrøm P, Gautvik KM. Expression and secretion of human parathyroid hormone in Saccharomyces cerevisiae. Gene, 90(2): 255-262, 1990.
3. Høgset A, Blingsmo OR, Sæther O, Gautvik VT, Holmgren E, Josephson S, Gabrielsen OS, Gordeladze JO, Alestrøm P, Gautvik KM. Expression and characterization of a recombinant human parathyroid hormone secreted by E.coli employing the staphylococcal protein A promoter and signal sequence. J. Biol. Chem., 265: 7338-7344, 1990.

In this regard we have received acceptance for an international patent on gene constructions, plasmids, the process and the down-stream technology.

In the further work we have by using in vitro mutagenesis, created full length parathyroid hormone agonist which has shown to be protease resistant and have interesting biological actions regarding mobilization of calcium from bone.

Both the intact hormone as well as the agonist will represent important medical drugs for use in diagnostics as well as represent a potential drug for treatment of various diseases.

4. Reppe, S., Olstad, O.K., Blingsmo, O.R., Gautvik, V.T., Sæther, O., Gabrielsen, O.S., Øyen, T.B., Gordeladze, J.O., Haflan, A.K., Tubb, R., Morrison, N., Tashjian, A.J. Jr., Alestrøm, P., Gautvik, K.M. Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product. ECB, 5th European Congress on Biotechnology, Copenhagen July 8-14, 1990. (Invited).
5. Reppe, S., Gabrielsen, O.S., Olstad, O.K., Morrison, N., Sæther, O., Blingsmo, O.R., Gautvik, V.T., Gordeladze, J.O., Haflan, A.K., Voelkel, E.F., Øyen, T.B., Tashjian Jr., and Gautvik, K.M. Production of recombinant human parathyroid hormone in yeast: Synthesis, purification, and biological characterization of a Lys-26-Gln site directed mutant. J. Biol. Chem., 266: 14198-14201, 1991.
6. Olstad, O.K., Reppe, S., Gabrielsen, O.S., Hartmanis, M., Blingsmo, O.R., Gautvik, V.T., Haflan, A.K., Christensen, T.B., Øyen, T.B., Gautvik, K.M. Isolation and characterization of two biologically active O-glycosylated forms of human parathyroid hormone produced in Saccharomyces cerevisiae. Identification of New Motif for O-glycosylation. Eur. J. Biochem., 205:311-319, 1992.
7. Forsberg, G., Brobjer, M., Holmgren, E., Bergdahl, K., Persson, P., Gautvik, K.M., and Hartmanis, M. Thrombin and H64A subtilisin cleavage of fusion proteins for preparation of human recombinant parathyroid hormone. J. Protein Chem., 1991, 10(5): 517-526.
8. Kareem, B.N., Rokkoknes, E., Høgset, A., Holmgren, E., Gautvik, K.M. A method for the evaluation of the efficiency of signal sequences for secretion and correct N-terminal processing of human parathyroid hormone produced in Escherichia coli. Analytical Biochemistry, 1992, 204: 26-33.

In our ongoing studies regarding the mapping of functional domains in human parathyroid hormone in comparison with the parathyroid hormone-like protein (PTHrP) we have expressed the human forms successfully in Saccharomyces cerevisiae and have also cDNA clones for their receptor as well as permanently transfected mammalian cells which express the receptor on the surface. By having access to PTH and PTH analogues as well as PTHrP, we are in a good position to map out the binding affinities of different hormonal forms as well as their coupling to different cellular signal systems.

Recently we have expressed the first known full length antagonist for hPTH, a long sought for molecule of considerable clinical interest. The compound has a binding K_D which is 2-4 times less than the natural hormone, but shows a more than 100-fold reduced biological activity.

9. Rian, E., Jemtland, R., Olstad, O.K., Gordeladze, J.O., Gautvik, K.M. Expression of biologically active human parathyroid hormone-related protein (1-141) in Saccharomyces cerevisiae. Eur. J. Biochem. 213: 641-648, 1993)
10. Rian, E., Jemtland, R., Olstad, O.Kr., Endresen, M.J., Grasser, W.A., Thiede, M.A., Henriksen, T., Bucht, E., Gautvik, K.M. Parathyroid hormone-related protein is produced by cultured endothelial cells; A possible role in angiogenesis. Biochem. Biophys. Res.

Commun., 198(2): 740-747, 1994.
11. Kareem, N.B., Rokkones, E., Høgset, A., Holmgren, E., Gautvik, K.M. Translocation and processing of various human parathyroid peptides in E.coli are differentially affected by protein A signal sequence mutation. Eur. J. Biochem, 220: 893-900, 1994.
12. Rokkones, E., Kareem, B.N., Olstad, O.K., Høgset, A., Schenstrøm, K., Hansson, L., Gautvik, K.M. Expression of human parathyroid hormone in mammalian cells, Escherichia coli and Saccharomyces cerevisiae. J. Biotechnol., 33: 293-306, 1994.
13. Olstad, O.K., Morrison, N.E., Jemtland, R., Jüppner, H., Segre, G.V., Gautvik, K.M. Differences in binding affinities of human PTH(1-84) do not alter biological potency: A comparison between chemically synthesized hormone and recombinant forms. Peptides, 15: 1261-1265, 1994.
14. Rokkones, E., Fromm, S.H., Kareem, N.B., Olstad, O.K., Høgset, A., Klungeland, H., Iversen, J., Bjøro, K., Gautvik, K.M. Human parathyroid hormone as a secretory peptide in milk of transgenic mice. Journal of Cellular Biochemistry, 59, 168-176 1995.
15. Olstad, O.K., Jemtland, R., Loseth, O.P., Bringhurst, F.R., Gautvik, K.M. Expression and characterization of a recombinant human parathyroid hormone partial agonist with antagonistic protperties: Gly-hPTH(-1→+84). Peptides, vol. 16, 1031-1037, 1995.
16. Mathavan, S., Gautvik, V.T., Rokkones, c., Olstad, O.K., Kareem, B.N., Maeda, S., Gautvik, K.M. High level production of human parathyroid hormone in Bombyx mori larvae and BmN cells using recombinant baculovirus. Gene, vol. 167, 33-39, 1995.
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18. Olstad O.K., Jemtland, R., Bringhurst, F.R., Gautvik, K.M. Expression and characterization of a partial recombinant human parathyroid hormone antagonist: Gly-hPTH(-1→+84). Manuscript, 1996.

The various recombinant PTH and PTHrP forms will be studied in their interaction with natural receptors of bone cells in culture and also in relation to recombinant receptor permanently transfected in mammalian cells. The aim of this is to understand in more detail the structure activity relationship between the different hormonal forms and their ability to activate different cellular signalling systems. The ultimate goal will be to try to understand how the osteoblast is activated by parathyroid hormone in the bone remocelling process of importance for the elucidation of the causes and patogenesis of osteoporosis.

II. NEUROENDOCRINE RECEPTORS AND THEIR FUNCTION

During our cDNA cloning of G-coupled receptors in rat pituitary cells and in human CNS, we have identified four potential candidates for G protein coupled receptors distinct from the TRH clone in a human phage library. We were able to isolate and characterize a functional human TRH receptor and to present these results as the first original international report. In addition to engaging in characterization of the other receptors, we are at present working on the organization and functional aspects of the gene for the human TRH receptor.

The ongoing and future research will concentrate on:

1. To map deleted receptor cDNA clones for functional activity using the Xenopus laevis oocyte system as a hormone (TRH) specific bioassay.
2. Generation of transfected cells to map out the hormone-binding receptor region as well as dissect which part of the hormone receptor couples to the two previously characterized G proteins which mediate signal system activation conveying its physiological actions, namely the Gαs coupling to the adenylyl cyclase system and the Gq/11 coupling to the phospholipase C.
3. Making hybrid receptors between the thyroliberin and the PTHrP/PTH receptor in order to analyze further the importance of the different receptor domain for conveying signal transduction.
4. Analyse the 5'-end of a newly isolated genomic clone for the human receptor.

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Two manuscripts in preparation.

III. STUDIES OF THE INTERACTION BETWEEN FATTY ACIDS AND DEXAMETHASOME AND INSULIN REGARDING REGULATION OF PEROXISOMAL ß-OXIDATION ENZYMES. POSSIBLE INVOLVEMENT OF THE PEROXISOMAL PROLIFERATOR ACTIVATED RECEPTOR (PPAR) GENE AND ITS REGULATION

On a collaborative basis within my institute, I have since 1991 engaged in research regarding hormonal control of peroxisomal ß-oxidation enzymes triggered by a surprising finding that fatty acids and dexamethasome have strong synergistic actions in regulation of the transcription of these three enzyme genes. This positive cooperativity was completely blocked by insulin in cultured liver cells and also in intact rats. The studies were carried out on the RNA, protein and enzyme activity levels and opened a new side of this already very much studied area of fatty acid ß oxidation. The ongoing research in this area will continue as a collaborative work between professor Jan-Åke Gustafsson's group at Huddinge Hospital and Institute for Medical Biochemistry, where we will concentrate on delineating the possible regulatory elements located in a genomic clone of the PPAR from rat.
Relevant O.A.: 120,125,127,131,147,150.

IV THE USE OF A NOVEL SENSITIVE MOLECULAR SUBTRACTION HYBRIDIZATION METHOD FOR STUDYING DIFFERENTIALLY EXPRESSED mRNAs

Studies of hypothalamic specific mRNAs obtained by subtraction hybridization procedure

Isolation and characterization of mRNAs specific for the osteosarcoma phenotype obtained by subtraction hybridization

Isolation and characterization of mRNAs psecific for parathyroid hormone gene activation in bone cells

Conclusion

This ongoing work has very successfully been able to isolate unique hypothalamic specific mRNAs among those also a novel somatostatin-like peptide. In addition, this subtracted library will probably contain the long sought for receptors which fatty acid or their metabolites are acting on, in order to regulate calorie intake and consumption. This bridges then the research going on in Chapter III and its work. The unique subtracted hypothalamic library can be exemplified with the finding that we also have isolated a novel calcium calmodulin kinase whose distribution is unique in CNS and also a transmembrane protein of secretory vesicles which has never previously been cloned in mammalian species, but has its homology in the electric organ of the electric eel. The characterization and studies of full-length cDNA clones from these mRNAs are given highest priority.

Already the differential display of mRNAs present in human osteosarcoma cells and absent in normal bone cells is very promissing and certainly leads to encouraging considerations regarding the possibility to obtain osteoporosis specific genes as defined by PTH specific mRNAs in normal osteoblasts.

PUBLICATION LIST

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Institute of Medical Biochemistry

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R/C 1

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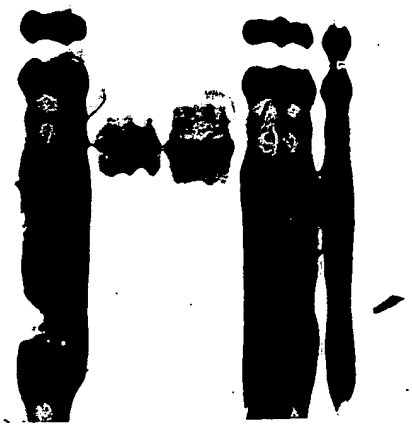

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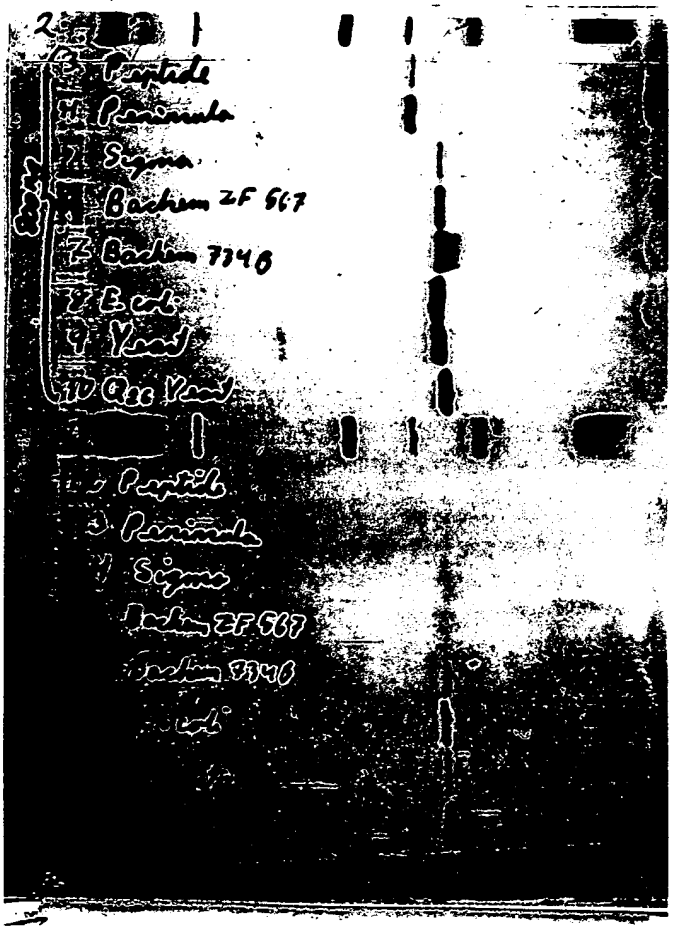

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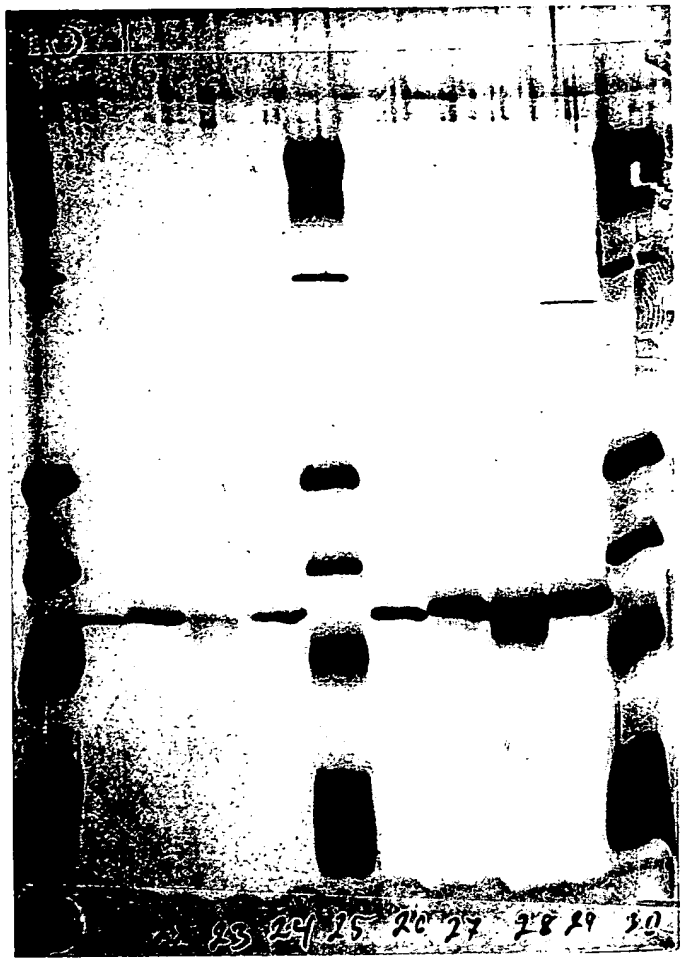

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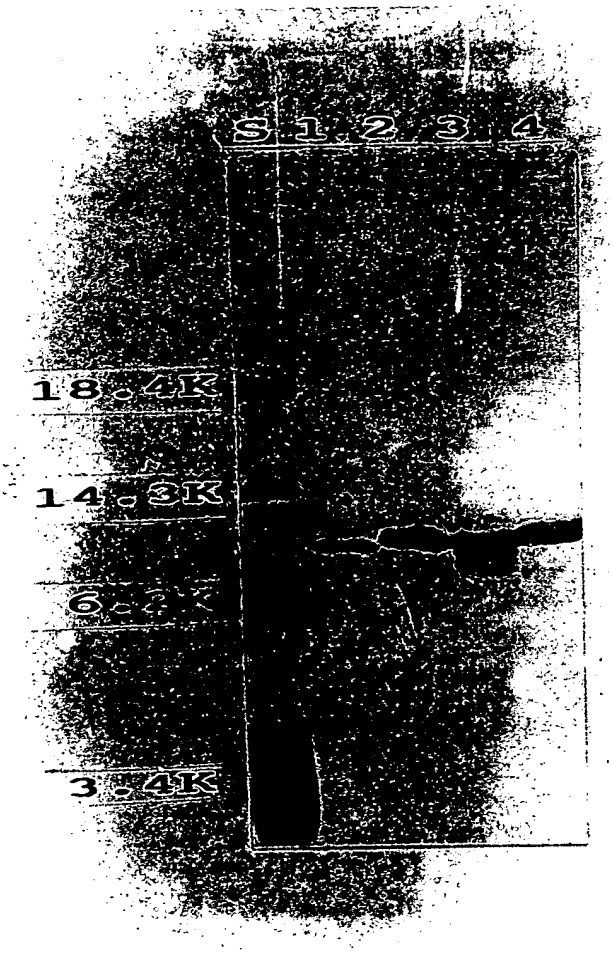

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Differences in Binding Affinities of Human PTH(1–84) Do Not Alter Biological Potency: A Comparison Between Chemically Synthesized Hormone, Natural and Mutant Forms

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OLSTAD, O. K., N. E. MORRISON, R. JEMTLAND, H. JÜPPNER, G. V. SEGRE and K. M. GAUTVIK. *Differences in binding affinities of human PTH(1–84) do not alter biological potency: A comparison between chemically synthesized hormone, natural and mutant forms.* PEPTIDES 15(7) 1261–1265, 1994.—The purpose of this study was to evaluate receptor binding affinities and biological properties in vitro and in vivo of various recombinant hPTH(1–84) forms representing the natural hormone and a mutagenized hPTH form, [Gln^{26}]hPTH(1–84) (QPTH), after expression in *E. coli* and *Saccharomyces cerevisiae*. In LLC-PK$_1$ cells stably transformed with the rat PTH/PTHrP receptor, chemically synthesized hPTH(1–84) and QPTH showed a reduced binding affinity (apparent K_d 18 and 23 n*M*, respectively) than the recombinant, hPTH(1–84) (apparent K_d 9.5 n*M*). All recombinant hPTH forms showed a similar potency to stimulate cellular cAMP production (EC_{50} 1.5 n*M*) and significantly better than chemically synthesized hPTH (EC_{50} 5.7 n*M*). All hormone forms showed an about equipotent activity in causing elevation in serum calcium, increased excretion of urine phosphate, and cAMP. Thus, the natural recombinant PTH forms showed higher binding affinities and adenylate cyclase activation potencies in LLC-PK$_1$ cells, but the reduced receptor binding affinity exerted by QPTH did not transcend differences in cAMP generation and in vivo biological activities.

Recombinant parathyroid hormones Recombinant PTH/PTHrP receptor cAMP response Rats

PARATHYROID hormone is the principle regulator of calcium homeostasis in humans and has been advanced as an anabolic drug against postmenopausal osteoporosis (22,25). The hormone, which is produced in the mammalian parathyroid glands, is synthesized as an 115 amino acid precursor that is processed to the mature hormone of 84 amino acids (21). The information required for high-affinity binding of PTH to its receptor in bone and kidney cells is contained within the biologically active 1–34 region (20). The amino-terminus of PTH is essential for triggering the adenylate cyclase response pathway (8,26), but it also contributes modestly to receptor binding affinity. In addition to a nearly complete loss of cAMP agonism, the deletion of residues 1–6 is accompanied by an approximately 100-fold decrease in receptor binding affinity (7,10,18,24). The major component of PTH receptor binding affinity, however, appears to be determined by residues 28–34. Deletion of these residues causes at least a 1000-fold reduction in binding affinity (18). Furthermore, PTH(25–34) displays weak, but detectable, receptor binding affinity ($K_d \approx 100$ μM) (18). In comparison, no evidence for receptor interaction has been obtained for amino-terminal fragments shorter than PTH(1–27) (24,26). Based on these observations, the 25–34 region has been called the hormone's principal receptor binding domain (18).

We have previously reported production of hPTH(1–84) in yeast (5), and the α-factor expression system is a well-characterized, commonly used strategy for expression of foreign proteins by the yeast *Saccharomyces cerevisiae* (3,27,29). The mating factor alpha (MFα) leader sequence is cleaved off sequentially by the KEX-2 endopeptidase and then by an amino peptidase STE13, leaving a correct *N*-terminal after guiding the recombinant protein through the secretory pathway (11). In the expression plasmid pαUXPTH-2, the MFα promoter, signal sequence, and termination signal were employed. The secreted hormone was purified from medium to more than 95% homo-

[1] Requests for reprints should be addressed to O. K. Olstad.

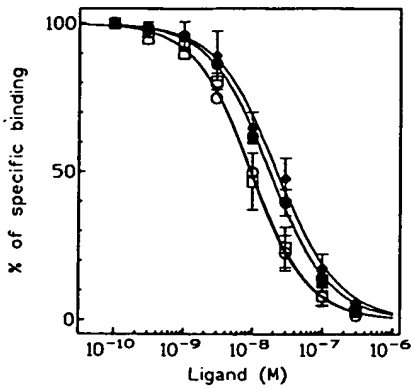

FIG. 1. Inhibition of radiolabeled [Tyr[36]]chickenPTHrP(1–36)amide by different hPTHs. Recombinant hPTH(1–84) produced in *E. coli* (○), recombinant hPTH(1–84) produced in *Saccharomyces cerevisiae* (□), [Gln[26]]hPTH(1–84) (QPTH) produced in *Saccharomyces cerevisiae* (♦), and chemically synthesized hPTH(1–84) (●) were tested in radioreceptor assay using LLC-PK$_1$ cells transfected with the rat PTH/PTHrP receptor. The data represent the mean ± SD of at last two independent experiments, each performed in triplicate.

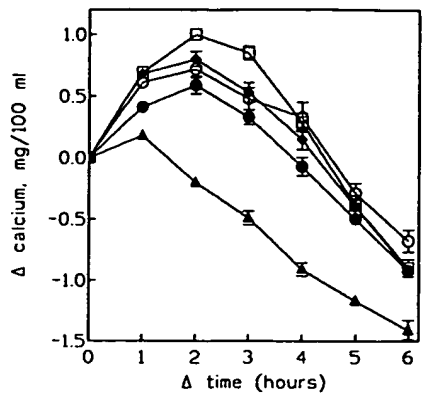

FIG. 3. Induction of hypercalcemia by different hPTHs. Parathyroidectomized male Wistar rats were administrated different forms of hPTH (2.0 μg of the recombinant hPTHs, 2.7 μg of the chemically synthesized hPTH) as described in the Method section. The stimulating agents were recombinant hPTH(1–84) produced in *E. coli* (○), recombinant hPTH(1–84) produced in *Saccharomyces cerevisiae* (□), [Gln[26]]hPTH(1–84) (QPTH) produced in *Saccharomyces cerevisiae* (♦), and chemically synthesized hPTH(1–84) (●). Control (▲). Blood samples were drawn at 0, 1, 2, 3, 4, 5, and 6 h after injection of PTH. The results are reported as the difference between the amount of calcium in the blood at the various time points, subtracting out the amount of calcium in the baseline sample (delta values). The data represents the mean ± SEM (n = 6).

geneity, characterized chemically, and shown to represent the natural hormone (5,19). In addition to the intact hormone, an aberrant KEX-2 cleavage occurring at an internal site (5) after two consecutive basic amino acids in the hPTH sequence -Arg[25]-Lys[26]↓Lys[27]-resulted in part fragmentation of the hormone. To improve the yield of hPTH, and to avoid internal degradation, a point mutation was introduced into the gene, changing Lys in position 26 to Gln (Q) (23). The resulting agonist, [Gln[26]]hPTH(1–84), called QPTH, was tested together with recombinant hPTH(1–84) produced in *Saccharomyces cerevisiae* and compared with chemically synthesized hPTH(1–84) in certain biochemical and biological tests.

We have also produced full-length hPTH in *E. coli* as a secretory product employing the *Staphylococcus aureus* protein A signal and regulatory sequences (9). After purification from medium and chemical characterization, this recombinant form was also included in the biochemical and biological characterizations.

LLC-PK$_1$ cells (porcine renal epithelial cells) stably transfected with the cDNA for the rat PTH/PTHrP receptor (4) were used for the receptor binding studies and cAMP responsiveness; rats

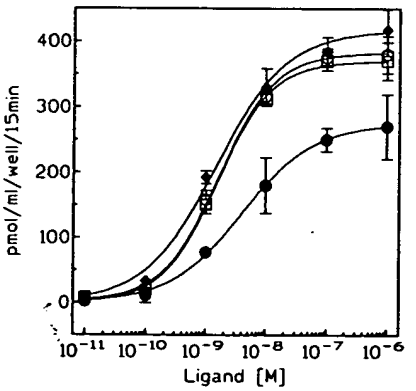

FIG. 2. Stimulation of cAMP by different hPTHs. Accumulation of intracellular cAMP in LLC-PK$_1$ cells transfected with the rat PTH/PTHrP receptor stimulated (15 min, 37°C) with recombinant hPTH(1–84) produced in *E. coli* (○), recombinant hPTH(1–84) produced in *Saccharomyces cerevisiae* (□), [Gln[26]]hPTH(1–84) (QPTH) produced in *Saccharomyces cerevisiae* (♦), and chemically synthesized hPTH(1–84) (●). The data represent the mean ± SD of two independent experiments, each performed in duplicate.

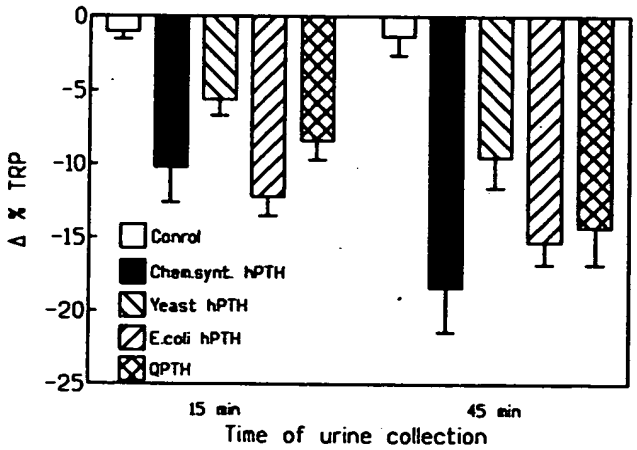

FIG. 4. Urinary excretion of phosphate. Parathyroidectomized male Wistar rats were administrated different forms of PTH (2.0 μg of the recombinant hPTHs, 2.7 μg of the chemically synthesized hPTH) as described in the Method section. Urine was collected for two periods: 0–30 and 30–60 min after administration of PTH. The excretion of phosphate is expressed as the percent tubular reabsorption of phosphate (% TRP) and is calculated by the formula: (1 − phosphate clearance/creatinine clearance) × 100. The result is reported as a change in % TRP related to the zero control level, and a decrease represents a greater amount of phosphate excreted in the urine. The data represents the mean ± SEM (n = 6).

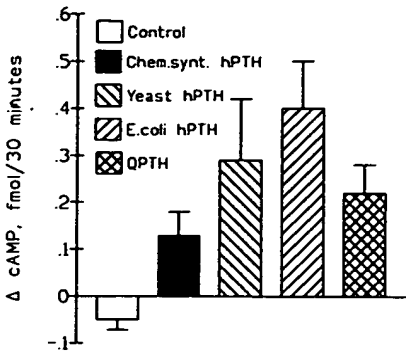

FIG. 5. Changes in urinary cAMP after administration of PTH. Parathyroidectomized male Wistar rats were administrated different forms of PTH (2.0 μg of the recombinant hPTHs, 2.7 μg of chemically synthesized hPTH) as described in the Method section. Urine was collected for 30 min after administration of PTH. The excretion of cAMP is reported as a change in cAMP concentration related to the zero control level. The data represents the mean ± SEM (n = 6).

were used for measurements of the hypercalcemic response, urine phosphate, and cAMP.

METHOD

Chemically synthesized hPTH(1-84) was purchased from Bachem Fine Chemicals (Torrance, CA) and [Tyr^{36}]chicken-PTHrP(1-36)-NH_2 for radioiodination was from Peninsula Laboratories. The production, purification, and chemical characterization of recombinant PTHs have been described previously (5,9,19,23). Peptide concentrations were determined by amino acid analysis. The blood and urine samples were analyzed for calcium, phosphate, protein, and creatinine on the Cobas Bio Autoanalyzer. cAMP was analyzed using a commercial radioimmunoassay kit from Amersham. All reagents were of highest purity available.

Radioreceptor Assay

LLC-PK_1 cells expressing the rat PTH/PTHrP receptor (4) were plated in 24-well plates. The cells were incubated with ^{125}I-labeled [Tyr^{36}]chickenPTHrP(1-36)-NH_2 (100,000 cpm per well/0.5 ml) in the presence or absence of competing ligands at 15°C for 4 h, using a Tris-based binding buffer (50 mM Tris-HCl), pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal calf serum as described (28). The competing ligands included chemically synthesized hPTH(1-84) from Bachem, recombinant hPTH(1-84) expressed in *E. coli* (9), recombinant hPTH(1-84) expressed in yeast (5,19), and recombinant QPTH expressed in yeast (23). Techniques used for radioiodination of PTHrP analogue have been reported (12,13). PTH and PTHrP bind to and activate PTH receptors in bone and kidney in an indistinguishable manner (12,14). [^{125}I][Tyr^{36}]chickenPTHrP(1-36)-NH_2 was used as radioligand because of lower nonspecific binding (less than 5% of total binding) (14) compared to [^{125}I][Nle^{8,18}Tyr^{34}]bovine-PTH(1-34)-NH_2, which gave 10-15% nonspecific binding (28).

Intracellular cAMP Measurements

For measurements of intracellular cAMP, LLC-PK_1 cells (4) expressing the rat PTH/PTHrP receptor were pleated in 24-well plates (50,000 cells/well) and grown to confluence for 3 days (about 250,000 cells/well). The cells were placed on ice, rinsed once with 1 ml of cold Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, and 0.1% bovine serum albumin. Medium (0.5 ml) with or without PTH was added and cells were transferred to a 37°C water bath for incubation in 15 min. Then the cells were rinsed once with 0.5 ml phosphate-buffered saline and immediately frozen on liquid nitrogen. Intracellular cAMP was measured by a radioimmunoassay kit from Amersham, after lysing the cells with 1 ml of 0.05 N HCl.

Hypercalcemic Assay

Male Wistar rats (150-200 g) were parathyroidectomized using electrocautery 18 h prior to the start of the experiment. Preliminary experiments showed that this was a reliable way to obtain complete removal of parathyroid gland activity because plasma calcium fell linearly as a function of time, as also indicated by the control group in Fig. 3. Moreover, individual rats showed small variations in the results. The parathyroid glands were removed for two reasons. One, to eliminate the endogenous production of the hormone, and two, to make the animals more sensitive to exogenous hormone. The increase in sensitivity is assumed to be due to the upregulation of PTH receptors in target organs (16). Thus, it has previously been shown that tubular membranes prepared from parathyroidectomized rats reveal a higher binding of [^{3}H]hPTH(1-34) and higher maximum stimulation of PTH-stimulated adenylate cyclase compared to control animals (16). It has also been shown (28) that downregulation of PTH receptors in ROS 17/2 cells occurs when the cells are exposed to PTH concentrations near hormonal physiological doses. More than 50% of the of the PTH-stimulated adenylate cyclase activity was recovered within 24 h after desensitization.

The animals were fasted overnight, and anesthetized the next day using hypnorm dormicum (0.2 ml/rat). The carotid artery was cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers acetate, 4 % bovine serum albumin, 25 units heparin/ml. Five minutes after injection of 200 μl of the heparinized Ringers acetate, a baseline blood sample was drawn (300 μl). The animals were tracheotomized to prevent respiratory failure due to damage to the recurrent laryngeal nerve running through the thyroid gland. The PTH agonist was then injected SC in a volume of 200 μl. All agonists were dissolved into 100 μl of 0.01 N acetic acid. The test agents included:

1. vehicle, 0.001 N acetic acid, 1% bovine serum albumin (control),
2. chemically synthesized human PTH(1-84), 2.7 μg/rat (chem. synt. hPTH),
3. recombinant human PTH(1-84) from yeast, 2.0 μg/rat (yeast hPTH),
4. recombinant human PTH(1-84) from *E. coli*, 2.0 μg/rat (*E. coli* hPTH),
5. recombinant [Gln^{26}]hPTH(1-84) from yeast, 2.0 μg/rat (QPTH).

Due to the reduced receptor binding affinity and cAMP stimulation in the in vitro assays below, the chemically synthesized hPTH concentration was used at 2.7μg/rat.

After dissolving in acetic acid, the agents were brought up in 900 μl of Ringers acetate containing 1% bovine serum albumin. Blood samples were drawn at 1, 2, 3, 4, 5, and 6 h after injection of hPTH or agonist. The rats were reheparinized 5 min before

drawing each blood sample using 200 μl of the heparinized Ringers solution.

All forms of hPTH were analyzed and quantified by amino acid analysis before administration to the rats.

The blood samples were centrifuged in a clinical centrifuge for 10 min, then the plasma was analyzed for calcium using a Cobas Autoanalyzer.

Urine Analysis

Male Wistar rats (150–200 g) were parathyroidectomized using electrocautery 18 h prior to the start of the experiment. The animals were fasted overnight, and anesthetized the next day using hypnorm dormicum (0.2 ml/rat). The carotid artery and the jugular vein were cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers acetate, 4% bovine serum albumin, 25 units heparin/ml. The bladder was catheterized using PE-200 tubing.

The carotid artery was cannulated for the collection of blood samples, and the jugular vein was cannulated for the purpose of injecting the hormones, and for a slow infusion for the purpose of volume loading the rats to increase the urine output. The rats were infused with Ringers acetate, 4% bovine serum albumin at the rate of 3 ml/h. The infusion was run for 2 h before the start of the experiment to equilibrate the animals.

After the 2-h equilibration period, a baseline urine collection was made for 30 min, with a midpoint arterial blood sample drawn at 15 min. At the end of the baseline urine collection, the PTH was injected IV, and a new 30-min urine collection was started. Again, a midpoint blood sample was taken 15 min into the urine collection. A final 30-min urine collection was made from 30–60 min after the injection of PTH, with the midpoint blood collection made at 45 min after PTH injection.

The excretion of phosphate is expressed as the percent tubular reabsorption of phosphate (% TRP). The % TRP is calculated by the formula: (1 − phosphate clearance/creatinine clearance) × 100. A decrease of % TRP represents a greater amount of phosphate excreted in the urine. The creatinine clearance did not change in any of the treatment groups. It was not expected to change, and was only measured to calculate the % TRP.

Statistical Analyses

A two-sample *t*-test was used comparing mean values of control and treated groups of animals (2).

RESULTS

Radioreceptor Binding Studies and Intracellular cAMP Measurements

Binding of the different hPTH forms is shown in terms of displacement curves using the [^{125}I][Tyr^{36}]chicken PTHrP(1–36)-NH_2 as radioligand and LLC-PK_1 cells permanently transfected with the rat PTH/PTHrP receptor.

The chemically synthesized hPTH and QPTH had calculated binding affinities with K_d of 18 n*M* (95% confidence interval: 16.1–20.0 n*M*) and 23 n*M* (95% confidence interval: 19.0–27.2 n*M*), respectively (Fig. 1). The natural recombinant hPTH(1–84) forms from *Saccharomyces cerevisiae* and *E. coli* had a similar but significantly lower apparent K_d of 9.5 n*M* (95% confidence interval: 8.7–10.4 n*M*) (Fig. 1). In spite of these differences in receptor binding affinities, all the recombinant hormones had equal ability to stimulate intracellular cAMP accumulation (EC_{50} about 1.5 n*M*, 95% confidence interval: 1.0–2.2 n*M*) (Fig. 2). In contrast, the synthetic hPTH showed a significant reduced potency to stimulate cAMP production with EC_{50} of 5.7 n*M* (95% confidence interval: 3.4–9.6 n*M*) on a molar basis, and a reduced maximal response.

Hypercalcemic Assay

After parathyroidectomy, the control calcium concentration fell linearly 1 h after the operation (about 0.75 mg %/h) (Fig. 3). The chemically synthesized hPTH was injected in a dose of 2.7 μg/rat compared to 2.0 μg/rat employed for the other hPTH species due to the reduced receptor binding affinity and cAMP stimulation of the chemically synthesized hPTH. These concentrations were chosen on the basis of preliminary experiments using a range of different doses and were selected because they gave a healthy hypercalcemic response and no observable side effects (e.g., unaffected rectal temperature). The hypercalcemic response of the chemically synthesized preparation was somewhat lower than for yeast hPTH, but almost similar to the other recombinant hPTHs. From these experiments it appeared that the declining parts of the curves were similar and like the slope of the control curve (Fig. 3).

Tubular Reabsorption of Phosphate

The percent tubular reabsorption of phosphate (% TRP) was calculated on basis of urine creatinine values and showed a strong and significant ($p < 0.01$) reduction after injection of the different PTH forms, and the potencies were similar. This effect was already observed 15 min after injection, and was then close to or at its maximum (Fig. 4). (For calculation of % TRP, the Method section.)

Measurements of Cyclic Adenosine Monophosphate

The changes in the cAMP content of the urine after administration of PTH was somewhat variable, with the chemically synthesized hPTH showing the smallest effect. However, all forms of PTH responded in a similar fashion. Therefore, there is no principal difference between the preparations of PTH in terms of their stimulation of cAMP release into the urine (Fig. 5).

DISCUSSION

Structural analysis of PTH indicates that PTH(19–34) fragment contains substantial helical structure (17) and the residues 17–28 form an α-helix (15). This assumption has been confirmed (6), showing that mutations of the hydrophobic residues Leu^{24}, Leu^{28}, and Val^{31} in hPTH are critical for optimal PTH activity, in contrast to most mutations of the polar residues (i.e., Lys^{26}, Lys^{27}, Gln^{29}, Asp^{30}, and His^{32}).

We have previously showed that QPTH is fully active in assays of adenylate cyclase, and this observation has been confirmed (6). Also in bone resorption studies using mouse calvaria (23), the QPTH was equally potent compared with the natural hormone. Biotinylation of Lys^{26} or Lys^{27} of [Nle^{8,18},Tyr^{34}]bPTH(1–34) has no effect on binding affinity (1), but substitutions as Lys^{26} → Glu and Lys^{26} → Thr causes partial reduction in cAMP production by PTH stimulation (6).

Interestingly, the substitution in QPTH, Lys^{26} → Gln, lowers the hormone's affinity to the receptor 2.4 times, but does not influence the cAMP production compared to the wild-type hormone, indicating that the efficacy of the hormone receptor complex to stimulate the cyclase dependent G-protein(s) may still be similar. This certainly also shows that it is important to complement receptor binding studies with functional analysis.

Our in vivo studies have shown that the recombinant forms of hPTH are at least as potent as chemically synthesized hPTH

(29% higher doses of the chemically synthesized preparation were used), demonstrating that the yeast and *E. coli* hPTHs were correctly processed and that the molecule folded correctly to give the proper tertiary structure, which is necessary to give full biological activity. Also, QPTH has folding characteristics that make it as active as the natural hormone. However, the reduced receptor binding potency and biological responses of the chemically synthesized hPTH on a molar basis is unexplained, but may be related to an inadequate *N*-terminal structure that is the last synthesized part of the peptide and for PTH is of crucial importance for receptor binding and eliciting the biological responses.

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